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Nuclear transfer of adult and genetically modified fetal cells of the rat

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Hayes, Eric, Sandra Galea, Amanda Verkuylen, Martin Pera, John Morrison, Orly Lacham-Kaplan, and Alan Trounson. Nuclear transfer of adult and genetically modified fetal cells of the rat. *Physiol Genomics* 5: 193–203, 2001.—The present study examines the handling, activation, and micromanipulation of rat eggs in an attempt to produce live young using nuclear transfer (NT) of adult and genetically modified rat fetal cells. Mature rat eggs cultured in calcium-free medium showed reduced rates (24%) of chromosomal dispersion ("spontaneous activation" characteristic of this species) compared with eggs cultured in calcium-containing medium (47%), but failed to survive micromanipulation procedures. High rates of parthenogenetic cleavage were obtained with chemical activation using ethanol/cycloheximide (65%) compared with other standard chemical activation methods (4–28%). This type of activation was also effective in reestablishing cleavage capability (19–71%), in a time-dependent manner, of spontaneously activated eggs arrested at a second prophase-like state. At most, two of four tested micromanipulation procedures were effective in producing NT embryos capable of morula or blastocyst development (14–16%) in vivo following transfer to mouse oviducts. NT blastocysts produced from cumulus cells and transfected rat fetal fibroblasts appeared morphologically and karyotypically normal ($2n = 42$). Nocodazole-assisted metaphase enucleation and piezoelectric-assisted donor cell injection produced significant and equivocal effects on survival and cleavage rates of reconstructed embryos but failed to significantly improve in vivo morula/blastocyst development rates (16–28%) compared with unassisted micromanipulation (16%). Live births have not yet been obtained from early cleavage stage embryos ($n = 269$) transferred to pseudopregnant recipient rat oviducts. Improvements in reconstituted NT embryo culture and transfer are required for these methods to be an effective means of transgenic rat production.

activation; transfection; embryo; oocyte; transgenic

MANY BIOMEDICAL RESEARCH FIELDS, including research in human diseases, use transgenic rats as an experimental model. Most transgenic rats have been produced by DNA microinjection into the pronuclei of fertilized oocytes with less than 20 transgenic rat lines produced (2). Recently, transgenic calves were obtained by nuclear transfer (NT) of genetically manipulated somatic cells (4). The use of NT of genetically modified somatic cells ensures that the genetic modification is carried in the germ line of offspring and therefore would be expected to be a more efficient method for production of stable transgenic lines. The generation of transgenic live young will therefore necessarily depend on the efficiency of the NT procedure. Live offspring have been obtained through NT of somatic cells in mice (26, 27), sheep (30), and cows (28). Attempts to produce rats from NT using embryonal blastomeres (11) and genetically modified fetal fibroblasts (8) have been unsuccessful thus far.

Efficient enucleation and artificial activation are essential components in the success of nuclear transplantation. The donor nuclear chromosomes replacing those of the metaphase II (MII) oocytes, undergo premature chromosome condensation and align in a metaphase-like configuration. To resume meiosis, form pronuclei, and initiate embryonic division cycles, NT oocytes require external activation stimuli (14, 26, 27). Mature superovulated rat oocytes exhibit a high rate of spontaneous activation (10) that results in an extrusion of a second polar body and scattered chromosomes in a stage known as metaphase III (MIII). When reaching this MIII state, oocytes exhibit very low rates of embryonic cleavage following chemically induced activation (32). Reduced numbers of normally fertilized rat oocytes following intracytoplasmic sperm injection has been attributed to this incomplete activation (7, 25) and may also be the limiting factor in the success of rat NT attempts described by Kono et al. (11) and Fitchev et al. (8).

The aims of the present study were to examine methods for rat oocyte handling, chemical activation, and micromanipulation for the production of NT rat embryos from adult somatic cells and genetically modified fetal cells.

METHODS

Animals. Female Sprague-Dawley rats (25–32 days old) were used as oocyte donors. Animals were maintained on a 12:12-h light-dark cycle (lights on 8:30 AM to 8:30 PM) and had access to food and water ad libitum.

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Media and supplements. All media, supplements, and drugs were obtained from Sigma Chemical (Sydney, NSW, Australia) unless indicated otherwise.

Oocyte collection and handling. Animals were injected subcutaneously with 20 IU pregnant mare serum gonadotrophin (PMSG; Intervet, Boxmeer, The Netherlands) and intraperitoneally with 15 IU human chorionic gonadotrophin (hCG; Inervet) 48–52 h and 12–14 h prior to oocyte collection, respectively (22). Animals were killed by decapitation, and the oviducts were removed in less than 5 min. Oviducts were collected into prewarmed calcium-free PBS. Oocytes were liberated from the oviducts into M16 culture medium containing 40 IU/ml hyaluronidase at 37°C using fine forceps. Oocytes were washed twice in M2 medium after 5-min exposure to hyaluronidase. Cumulus-free oocytes were transferred to equilibrated M16 medium and incubated in humidified 5% CO_2 in air at 37°C until use (29). Oocytes were not scored prior to allocation and were distributed at random to activation treatment groups.

Spontaneous activation. Oocytes were placed into prewarmed and equilibrated calcium-free M16 or calcium-containing M16 medium for 4–8 h. Oocytes were classified as metaphase I (MI; no polar body), metaphase II (MII; 1st polar body extruded), metaphase III (MIII; 2nd polar body extruded) or other (e.g., cleaved, fragmented, lysed). To determine the status of the nuclear DNA in MII and MIII oocytes, samples from each group were fixed in PBS containing formaldehyde (1%), glutaraldehyde (0.2%), and FCS (1%). Fixed embryos were stained with Hoechst 12387 (10 μM) and visualized under ultraviolet (UV) light at ×40 magnification. Images were captured and digitized on PC using software designed for a Leica microscope (model DMR for epifluorescence).

Chemical activation. All activation reagents except ethanol were prepared in M16. Ethanol was prepared in PBS supplemented with 4 mg/ml BSA. Oocytes were allocated to one of 5 activation protocols: *1*) ethanol (8%; 5 min) and cycloheximide (35 μM; 4 h), *2*) calcium ionophore (A23187, 5 μM; 5 min) and dimethylaminopyridine (DMAP; 2 mM; 4 h), *3*) strontium (10 μM; 4 h), *4*) thimerosal (10–100 μM; 15–30 min), or *5*) M16 alone. All oocytes were cultured in M16 medium following the last step in activation. The status of oocytes was recorded 24 h later as MI (no polar body), MII (1 polar body extruded), MIII (2 polar bodies extruded), cleaved (2 cell), or other (>2 cell, fragmented, lysed).

Diploid parthenotes and rescue. In an attempt to induce diploid parthenogenetic development in vitro, oocytes were activated with ethanol followed by treatment with cycloheximide in the presence of cytochalasin B (5 μM). In a separate experiment, "rescue" of spontaneously activated oocytes with ethanol and cycloheximide at 4 and 8 h after collection from rat oviducts was attempted. Oocytes exhibiting a second polar body at 4 or 8 h after collection were activated with ethanol followed by cycloheximide as described above. These oocytes were cultured in M16 medium and observed 24 and 48 h later for parthenogenetic development as described above.

Fibroblast culture and transfection. Primary rat embryonic fibroblasts (REFs) were isolated from fetuses obtained from females on *day 13.5* to *day 14.5* of pregnancy. Cells were produced through digestion of fetal carcasses (following removal of head and organs) in PBS containing penicillin (200 IU/ml), streptomycin (200 μg/ml), trypsin (0.1% wt/vol), and EDTA (0.1% wt/vol). Washed cells were plated at a density of 5–7 × 10^6 cells/ml in 50-ml flasks (Falcon; Becton-Dickinson, Melbourne, VIC) and cultured in DMEM (GIBCO, Life Technologies, Sydney, NSW) supplemented with 10% FCS (GIBCO). Cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air (6).

The lacZ-neomycin reporter construct (pcDNA-His-lacZ) was obtained from Invitrogen (Groningen, The Netherlands) and green fluorescent protein-hygromycin (pHygEGFP) reporter construct was obtained from Clontech (Palo Alto, CA). For stable transfection experiments, the lacZ vector was linearized with *Bgl*II, and the GFP vector was linearized with *Xmn*I. Transfection experiments were initiated on *day 3* of culture in 10-cm dishes using Lipofectamine Plus. Transfection involved addition of 8 μg of linearized plasmid to 20 μl of Plus reagent in 750 μl of serum-free (SF) media with incubation at 23°C for 15 min. Lipofectamine, 30 μl, was then added to 720 μl of SF media, and the solutions were then mixed together and incubated at 23°C for a further 15 min. Media was then aspirated from the cells and replaced with 5 ml of SF media. The DNA/Lipofectamine solution was then added to the cells followed by the addition of 6.5 ml of DMEM containing 10% FCS 2–3 h later. On the following day, media was replaced with DMEM/10% FCS containing either 300 μg/ml of Geneticin (Life Technologies, Sydney, NSW) for the lacZ reporter construct or 50 μg/ml of hygromycin for the GFP reporter construct. Antibiotic selection was continued for a period of 10 days (i.e., *day 14*). Transfected cells from *days 14–21* were used in NT experiments.

Nuclear transfer. Four different NT models were used with one of two chemical activation protocols consisting of strontium (10 μM, 4 h) or ethanol (8%, 5 min)/cycloheximide (35 μM, 4 h). Cumulus cells were collected from oocytes after hyaluronidase treatment. Cumulus cells were washed and stored in 40 μl of equilibrated M16 medium until use (less than 30 min). Transfected fetal fibroblasts were lifted from culture flasks through trypsin digestion (0.025%), washed in DMEM containing soybean trypsin inhibitor (1 mg/ml), and resuspended at a concentration of 0.5–1.0 × 10^6 cells/ml in PBS containing 4 mg/ml BSA. Immediately prior to NT experiments, cumulus cells and/or transfected fibroblasts (15–20 μl) were resuspended in 50 μl methylcellulose (MC) or polyvinylpyrrolidone (PVP) prepared in M2 medium (2% wt/vol).

The first model (*model A*) consisted of removal of the metaphase plate of MII oocytes collected 12 h after hCG following removal of cumulus cells with hyaluronidase (40 IU/ml). Mechanical enucleation of the polar body and metaphase plate was accomplished with a micropipette having an internal diameter of 7–10 μm. Enucleated oocytes were washed once in equilibrated M16 medium and cultured for 1–2 h prior to injection of a donor cell. The reconstructed oocyte was activated 30 min to 1 h after donor cell injection. In cases where nocodazole-assisted mechanical enucleation and piezoelectric-assisted donor cell injection were employed, *model A* was used with slight modification. Briefly, cumulus-free oocytes were transferred to M16 culture drops containing 10 μM nocodazole for 1 h prior to enucleation. Oocytes were washed twice in equilibrated M16 medium prior to enucleation of the metaphase cone in 100-μl drops of M2 handling medium. Oocytes were washed once in equilibrated M16 medium and cultured for 1–2 h before piezoelectric-assisted donor cell injection. Injections were performed using a blunt micropipette with an internal diameter of 5 μm. Donor cumulus cells were loaded into the pipette, and the pipette tip was placed against the zona pellucida of enucleated oocytes. Following application of a slight negative pressure through the injection pipette, two to three pulses were delivered to bore a hole in the zona pellucida. The pipette was then advanced into the perivitelline space while the cell was gently extruded to the point of making contact with the

oolemma. Donor cells were deposited in the ooplasm after application of negative pressure through the injection pipette followed by a single pulse of reduced intensity to break the oolemma. Reconstructed embryos were chemically activated as described above at 30 min to 1 h after donor cell injection.

The second model (*model B*) involved mechanical injection of donor cells into MII oocytes followed by chemical activation 1 h later. The female pronucleus (PN) was then removed by enucleation at the time of its appearance (~6 h after donor cell injection). The third model (*model C*) involved chemical activation of MII oocytes as described above followed by mechanical injection of donor cells 1 h later and subsequent removal of the female PN (~4 h after activation). The female PN was easily distinguished by its proximity to the second polar body. Enucleation was not undertaken when the position of the female PN was questionable. The fourth model (*model D*) involved chemical activation followed by the removal of the female PN (~4 h after activation). A donor cell was then injected immediately after enucleation of the female PN. Mature oocytes were held in PBS supplemented with 4 mg/ml BSA in all NT models except *model C*, where activated oocytes were held in PBS plus BSA containing 35 μM cycloheximide. All enucleations were carried out in PBS plus BSA containing 5 μg/ml cytochalasin B.

Reconstituted oocytes were cultured for 24–48 h in M16 medium and were transferred to the oviducts of pseudopregnant female mice (*day 1*) or rats (*day 1*) at the one- to four-cell stage. Mice were made pseudopregnant by pairing with a vasectomized male 24 h prior to embryo transfer (3). Rats were made pseudopregnant by mechanical stimulation of the cervix 24 h prior to embryo transfer (18). The reproductive tracts of recipient mice were flushed at 3–4 days after transfer and examined for the presence of morulae or blastocysts. Recovered morulae were cultured in M16 medium for a further 24 h. Rats that did not deliver live young were killed between 22 and 26 days after transfer to examine the uterus for signs of implantation.

Embryo staining and karyotype analysis. To visualize cell development in NT embryos collected from recipient mice, embryos were fixed in PBS containing formaldehyde (1%), glutaraldehyde (0.2%), and FCS (1%). Fixed embryos were stained with Hoechst 12387 (10 μM) and visualized under UV light at ×40 magnification. Images were captured and digitized on PC using software designed for a Leica microscope (model DMR for epifluorescence). Metaphase spreads used for karyotype analysis were prepared according to the technique developed by Michaeli et al. (17) with minor modifications. Nuclei were arrested at the metaphase stage by culturing embryos in 100 μg/ml of colchicine for 8–10 h. Embryos were then incubated in 1.0% sodium citrate solution on ice for 30 min and transferred in a small drop of sodium citrate to a grease-free slide. The embryos were spread using a 1:1 solution of glacial acetic acid and methanol followed by fixing with a 1:3 solution of glacial acetic acid and methanol. The slides were air-dried, and the preparations were stained with 10% Giemsa. Chromosome preparations were observed at ×1,000 magnification, assessed using the Analytical Imaging Station program and Fujix HC-1000 camera, and scored for ploidy.

Statistics. Data for rat oocytes subject to spontaneous activation and rescue after spontaneous activation were analyzed by χ^2. Data obtained after chemically induced activation of rat oocytes were analyzed using Kruskal-Wallis ANOVA for nonparametric data followed by Cochran's Q test for multiple comparisons. Data for NT models within a given nuclear donor cell type were analyzed by Fischer exact test for proportions. $P < 0.05$ was taken as a significant result in all cases.

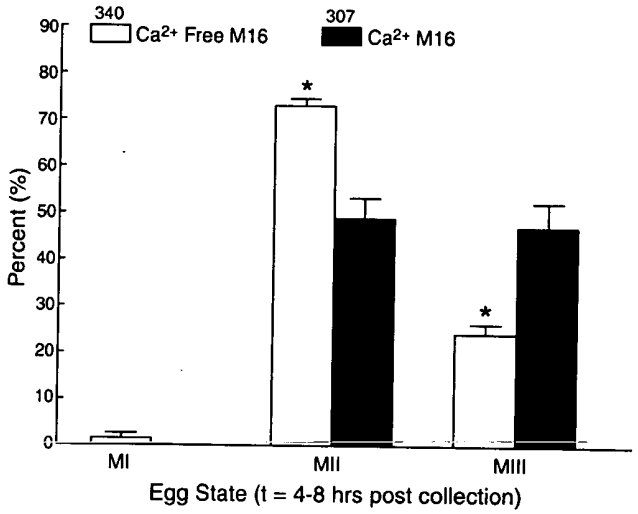


Fig. 1. Effects of 4–8 h of culture in calcium-free (open bars) or calcium-containing culture medium (solid bars) on progression of superovulated oocytes to a second prophase-like abortive state. Oocyte status following culture was described as metaphase I (MI; no polar body extruded), metaphase II (MII; one extruded polar body), or metaphase III (MIII; two extruded polar bodies). *Significant difference compared with calcium-containing M16.

RESULTS

Spontaneous activation. After 4–8 h in calcium-containing M16 culture medium, 49 ± 4.4% of oocytes remained in the MII state, whereas 47.2 ± 5.2% of oocytes progressed to an MIII state (Fig. 1). Oocytes in the MII state exhibited a clearly defined metaphase plate (Fig. 2*A*), whereas oocytes that had progressed to the MIII state exhibited varying degrees of DNA dispersion (Fig. 2*B*). Oocytes cultured in calcium-free M16 medium for 4–8 h tended to remain in an MII state (73.1 ± 1.6%; $P < 0.001$ vs. M16 medium containing calcium) with fewer oocytes progressing to an MIII state (24.3 ± 2.0%; $P < 0.005$ vs. normal M16) (Fig. 1). Oocytes handled in calcium-free M16 medium appeared granular and failed to survive micromanipulation of any kind (Fig. 2*C*), despite less variation in chromosomal dispersion.

Chemical activation. The number of oocytes remaining at the MI stage after chemical activation and 24 h of culture in M16 medium was not significantly different across treatment groups (Table 1; $P = 0.49$). A significant number of control oocytes remained in an MII state compared with all other activation methods (Table 1; $P < 0.001$). A trend was observed for control oocytes, as well as oocytes treated with strontium and thimerosal, to progress to an MIII state compared with ethanol/cycloheximide and calcium ionophore/DMAP activated oocytes, but this effect was not statistically reliable (Table 1; $P = 0.12$). Activation of rat oocytes with ethanol/cycloheximide and calcium ionophore/DMAP produced significantly larger numbers of two-cell parthenotes compared with other chemical activa-

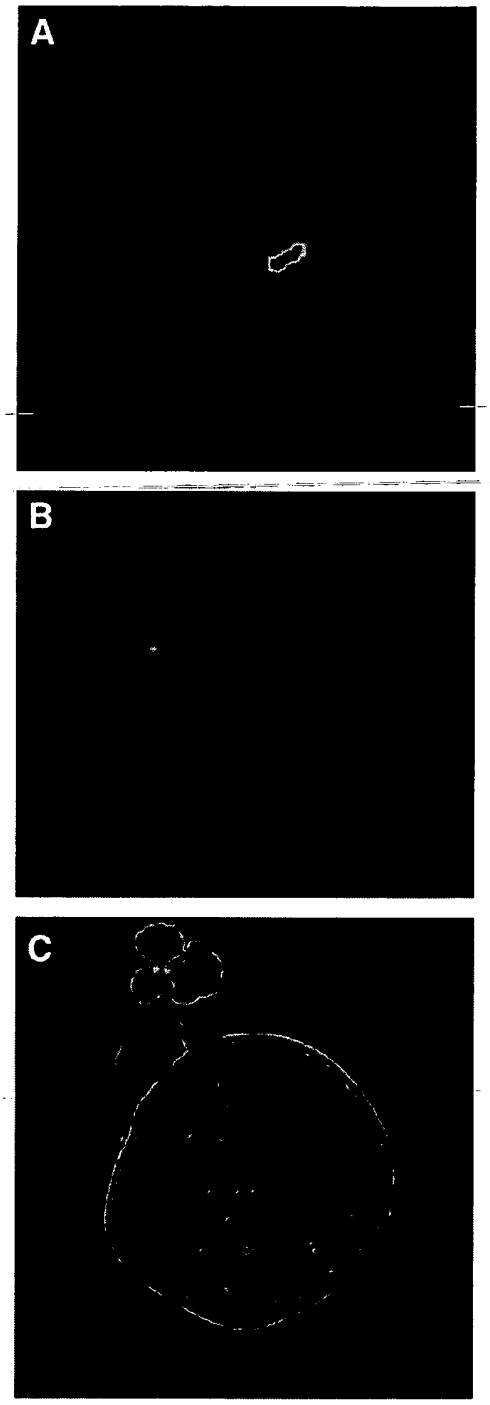


Fig. 2. Fluorescent image of an MII rat oocyte cultured in calcium-free M16 medium (*A*) or calcium-containing M16 medium (*B*) for 4–8 h after collection. The embryos were stained with 10 μM Hoechst 12387, visualized at an original magnification of ×40, and captured as a digitized image using PC-based software designed for a DMR model Leica microscope. *C*: a light microscopic image of an MII rat oocyte cultured in calcium-free M16 medium for 4–8 h after collection. The embryo was visualized at an original magnification of ×40.

tion methods and controls (Table 1; $P < 0.001$). Ethanol/cycloheximide produced 64.5 ± 10.9% two-cell parthenotes, an effect significantly greater than 27.7 ± 5.5% parthenotes produced by calcium ionophore/DMAP. Rat oocytes activated in ethanol/cycloheximide exhibited better blastomere morphology and advanced cell division at 24 h after activation compared with those activated in strontium or thimerosal (Fig. 3, *A–C*).

Diploid parthenotes and rescue. Activation of rat eggs with ethanol/cycloheximide in the presence of cytochalasin B produced two-cell parthenotes at a rate of 89.8 ± 3.4%, a rate much higher than the same activation in the absence of cytochalasin B (64.5 ± 10.9%; Table 1). The ethanol/cycloheximide activation method was able to rescue parthenogenetic development when applied to spontaneously activated rat eggs (i.e., extruded second polar body) at 4 h and 8 h post-collection. Oocytes that were chemically activated at 4 h after spontaneous activation cleaved at significantly ($P < 0.05$) higher rates (42% and 70.7% cleavage at 24 and 48 h after chemical activation, respectively; $n = 205$) compared with oocytes chemically activated at 8 h after spontaneous activation (19.3% and 26.4% cleavage at 24 and 48 h after chemical activation, respectively; $n = 197$).

Fibroblast culture and transfection. REFs were transfected with both lacZ and GFP. The lacZ was initially used to optimize the transfection conditions and to obtain stable cell lines that were clonal. This process took 4–6 wk, by which time the fibroblasts had undergone senescence. Continued culturing of the cells resulted in a reinitiation of mitosis; however, this was presumably due to the cells going through "crisis" and hence being transformed (23). We have made the assumption that transformed cells are not suitable for cloning. To avoid using transformed cells, strategies were developed for transfecting cells within 2 wk. This required shortening the initial culturing period after isolation of the cells to 2 days and reducing the time used for Geneticin selection to 10 days. Hence, cells could be transfected with lacZ or GFP and used in NT experiments within 2 wk of isolation. This strategy does not produce clonal cell lines, nor were the transfection rates 100%; however, the cells produced by this method proved suitable for these studies.

Nuclear transfer. The efficiencies for the four NT protocols using adult somatic cells and genetically modified fetal cells as donor nuclei are shown in Table 2. Cleavage rates of NT embryos produced by cumulus and transfected fetal fibroblast cells (11.7% and 22.6%, respectively) were significantly ($P < 0.05$) reduced compared with cleavage of parthenogenetic controls (55.2% to 67.6%). Overall, of 4,720 and 1,256 oocytes manipulated and 1,935 and 292 oocytes injected with cumulus and transfected fibroblasts, respectively, development rates to morula or blastocyst for reconstituted embryos transferred to mouse oviducts were not significantly different (16.6% vs. 14.3%). Reconstituted embryos produced from either cell type appeared morphologically normal (Fig. 4, *A* and *B*) and karyotypi-

Table 1. *Oocyte responses to chemical activation*

Response of Oocyte to Activation	Method Used for Chemical Activation of Oocytes				
	EtOH/Cycloheximide	CaI/DMAP	Strontium	Thimerosal	M16
MI	0.2 ± 0.2^a	0 ± 0^a	0 ± 0^a	0.8 ± 0.8^a	1.0 ± 0.8^a
MII	8.2 ± 4.4^a	4.5 ± 2.3^a	4.6 ± 2.0^a	17.7 ± 8.2^a	33.7 ± 4.1^b
MIII	14.8 ± 8.3^a	14.8 ± 8.6^a	53.7 ± 19.4^b	35.0 ± 16.2^b	19.3 ± 4.5^a
Cleaved	64.5 ± 10.9^b	27.7 ± 5.5^b	3.8 ± 3.0^a	3.6 ± 2.3^a	12.8 ± 3.1^a
Other	12.3 ± 4.6^b	53.0 ± 9.9^a	38.0 ± 17.8^a	42.8 ± 14.2^a	33.2 ± 3.2^a
Total oocytes	322	337	258	226	312

Values are means ± SE and are expressed as percentage of oocytes, which describe the effects of different chemical activation protocols on rat parthenogenote development in vitro. EtOH, ethanol; CaI, calcium ionophore; DMAP, dimethylaminopyridine. Different superscript letters (a and b) indicate significant differences between columns. The "Response of Oocyte to Activation" was evaluated at 24 h following chemical activation and culture in M16 medium.

cally normal (Fig. 5, *A* and *B*). Following Hoechst staining, reconstituted embryos derived from cumulus cells exhibited high cell numbers consistent with good blastomere morphology and normal karyotype (Fig. 6, *A* and *B*). However, neither donor cell type was capable of full-term development following transfer of reconstituted embryos to pseudopregnant female rats (*model A* only).

The NT efficiencies for both nuclear donor cell types are shown according to method in Tables 3 and 4. Despite lower overall survival rates (survived to culture/total manipulated) for oocytes receiving transfected fibroblasts, survival for oocytes receiving cumulus cells or transfected fibroblasts, respectively, was consistently higher for *model A* (24% and 10.1%), *model C* (23% and 11.0%), and *model D* (24% and 14.6%) compared with *model B* (6.0% and 5.1%). For reconstituted embryos derived from cumulus cells, cleavage rates were highest for *model B* (36.1%) and *model C* (31.2%), with lower cleavage rates in *model A* (5.5%) and *model D* (0.0%). Cleavage rates for embryos derived from transfected fibroblasts were similar (20–33%) regardless of the model employed. Cleavage rates of cumulus cell and transfected fibroblast-derived NT embryos were significantly ($P < 0.05$) lower than parthenogenetic cleavage rates (51.5% to 85%). Development of cumulus cell-derived embryos to morula/blastocyst in mouse oviducts occurred for *models A* and *B* only (18.8% and 33.0% of transferred embryos, respectively). To date, we have observed the development of only a single blastocyst using transfected rat fetal fibroblasts as nuclear donors (*model C*).

Table 2. *Overall nuclear transfer efficiencies*

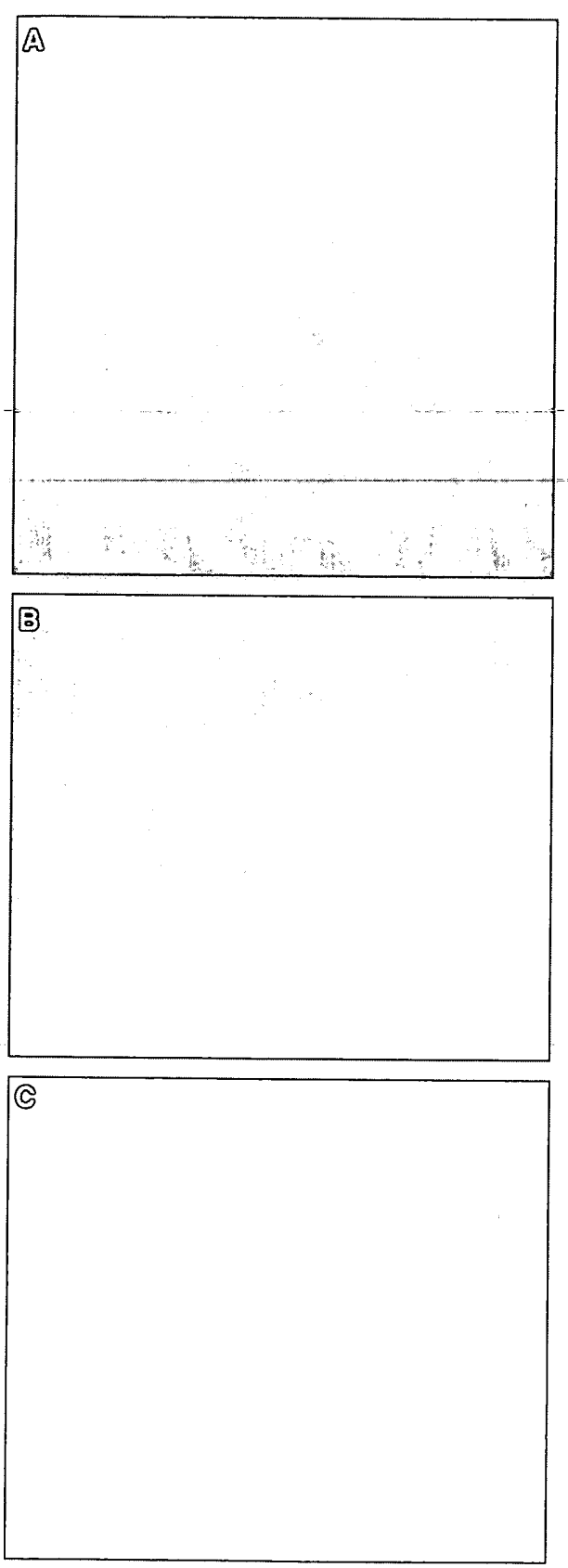
	Donor Cell Type	
	Cumulus Cell	Transfected Embryonic Fibroblast
Oocytes		
First manipulation	4,720	1,256
Survived	2,548	441
Second manipulation	1,935	292
Survived	862(18.3)[a]	106(8.4)[b]
Cleaved	101(11.7)[a]	24(22.6)[b]
Embryos		
Transferred to mice	193	7
Transferred to rats	269	NA
Developing to morula/blastocyst	32(16.6)[a]	1(14.3)[a]
Producing live born	0(0.0)	NA

Values describe overall nuclear transfer efficiency according to donor cell type independent of model employed. Values in parentheses are in percent. First manipulation indicates enucleation of the metaphase plate or donor cell injection or removal of the female pronucleus, respectively, followed by donor cell injection or enucleation of the female pronucleus or donor cell injection, respectively (second manipulation). Significant differences in reconstructed embryo survival, cleavage, and development in vivo between donor cell types are indicated by different superscript letters (a and b). NA, not applicable.

Model A was employed in an attempt to determine the utility of chemical stabilization of the metaphase plate for mechanical enucleation, and piezoelectric-assisted donor cell injection, on NT procedures using cumulus cells. Overall survival rates for enucleated oocytes were 6.7% lower for nonpiezoelectric-assisted cell injections compared with piezoelectric-assisted cell injections ($P < 0.05$; Table 5). Nocodazole-assisted enucleation produced a significant reduction of 7.5% in oocyte survival compared with blind enucleation ($P < 0.05$; Table 5). Therefore, blinded enucleation and piezoelectric-assisted donor cell injection produced the best overall oocyte survival rates (29.9%). Oocytes enucleated following nocodazole treatment appeared to cleave at significantly higher rates than those enucleated blindly ($P < 0.05$; Table 5). Cleavage rates were not significantly different for nonpiezoelectric- and piezoelectric-assisted donor cell injection. Despite higher cleavage rates in nocodazole-treated oocytes, development rates in mouse oviducts did not differ significantly between reconstituted embryos derived from oocytes enucleated in the presence (16/75, 21.3%) or absence (13/79, 16.4%) of nocodazole. In vivo development rates in mouse oviducts were also not significantly different for nonpiezoelectric-assisted (15/74, 20.2%) and piezoelectric-assisted donor cell injections (14/80, 17.5%).

DISCUSSION

The factors involved in spontaneous activation of rat oocytes are believed to be *1*) the time oocytes are left in the oviducts before retrieval from the source animal and *2*) the temperature used for oocyte manipulation following retrieval (10, 32). Reducing the time of oocyte



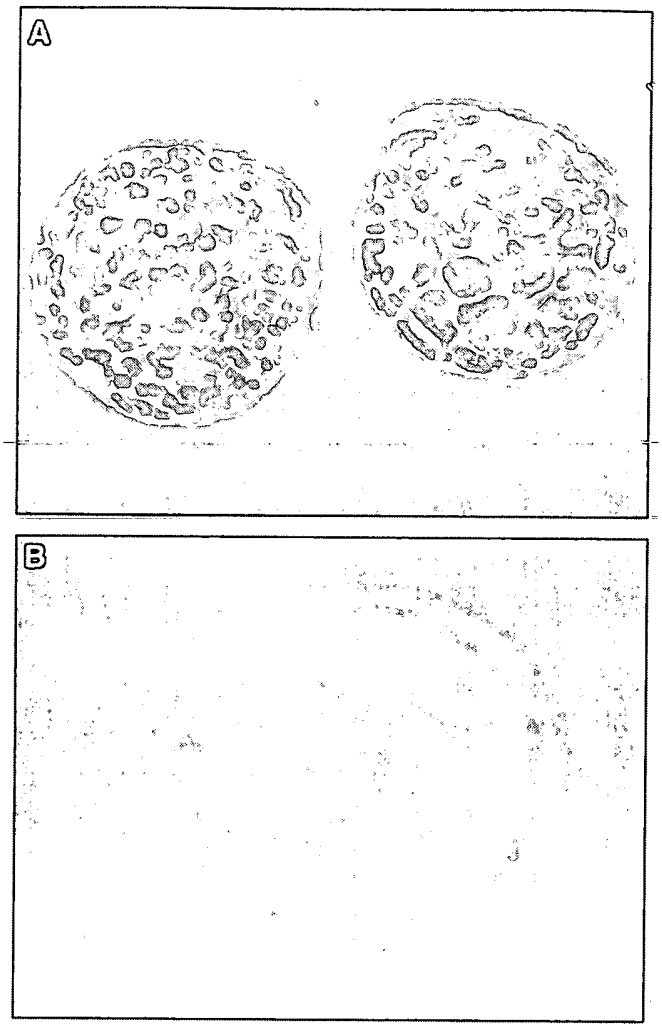


Fig. 4. Light microscopic images of nuclear transfer (NT) blastocysts derived from cumulus cells (*A*) and transfected fetal fibroblasts (*B*) and recovered from recipient mouse oviducts at 3–4 days following transfer.

retrieval to less than 5 min from death and handling of oocytes at 37°C reduces spontaneous activation of rat oocytes (32). To prevent spontaneous activation, even after rapid removal of eggs from the source animal, we attempted to maintain the oocyte in an MII static state using calcium-free media for oocyte collection and micromanipulation. Despite reduced migration of the metaphase plate in oocytes handled in calcium-free medium, these oocytes failed to survive any form of attempted micromanipulation. The rat oocyte may have a limited capability to respond to widely variable calcium ion concentrations during medium changes

Fig. 3. Light microscopic image of a parthenogenetic rat oocyte activated with ethanol (8%, 5 min) and cycloheximide (35 μM; 4 h) (*A*), calcium ionophore (5 μM, 5 min) and dimethylaminopyridine (2 mM; 4 h) (*B*), and strontium (10–100 μM, 4 h) (*C*). Embryos were cultured in M16 medium for 24 h after activation and were visualized at an original magnification of ×40.

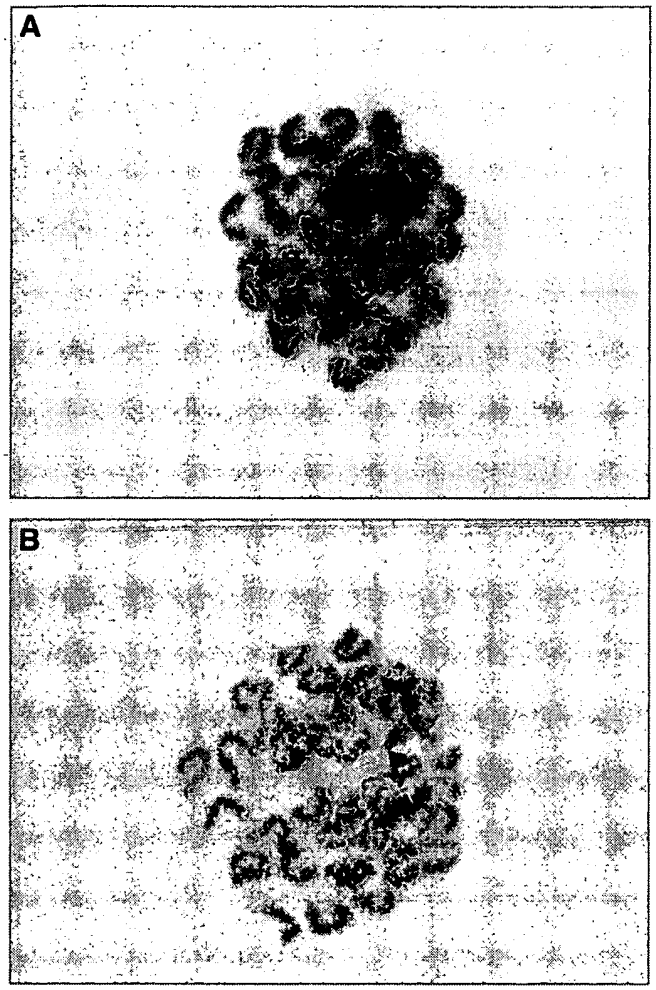


Fig. 5. Light microscopic images (original magnification, ×1,000) of karyotypes obtained from NT blastocysts derived from cumulus cells (*A*) and transfected fetal fibroblasts (*B*) and recovered from recipient mouse oviducts at 3–4 days following transfer.

and appears to be particularly sensitive to the absence of calcium ions in the handling medium. Given these observations, rapid removal from the source animal and maintenance of oocytes at 37°C appears to be the most efficient system for obtaining rat oocytes suitable for micromanipulation procedures, despite relatively high rates of spontaneous activation.

Oocytes maintained in culture medium in the absence of chemical activation exhibited limited rates of cleavage consistent with previous studies in this species (32). The reasons for the high rate of arrest at a MIII state in oocytes activated with strontium and thimerosal are not known, as both compounds have been used successfully in other species (13, 15, 21). O'Neill et al. (21) reported that exposure of mouse oocytes to strontium in M16 for longer than 10 min produced a lower rate of activation and a higher rate of oocyte degeneration. We (19) and others (13) have used strontium successfully for activation of mouse oocytes at durations equal to or greater than the duration used in this study (4–8 h). It is interesting to note that Chinese hamster oocytes are refractory to activation induced by strontium (24), thus suggesting a possible species effect for oocyte responsiveness to strontium. Machaty et al. (16) have recently reported that thimerosal-induced activation is effective for porcine oocytes but only in the presence of dithiothreitol (DTT). This may be a very important means of chemical activation, as thimerosal-DTT appears to induce calcium oscillations of similar magnitude and frequency compared with calcium oscillations produced by fertilization with spermatozoa (1). The highest proportions of artificially activated rat oocytes which underwent the first and second cleavage cycles in culture were found in the group treated with ethanol followed by exposure to the protein synthesis inhibitor cycloheximide. These results are consistent with our previous experience in using this type of chemical activation in other species

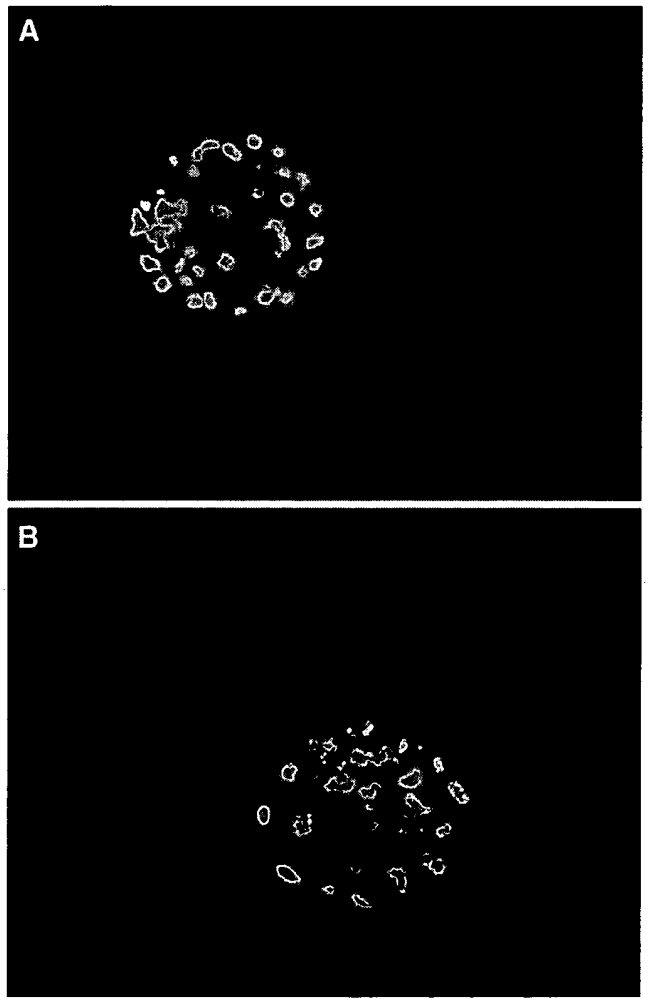


Fig. 6. Fluorescent microscopic images of cell distributions in NT blastocysts derived from cumulus cells (*A*) and transfected fetal fibroblasts (*B*) and recovered from recipient mouse oviducts at 3–4 days following transfer. The embryos were fixed and stained with 10 μM Hoechst 12387, visualized at an original magnification of ×40, and captured as a digitized image using PC-based software designed for a DMR model Leica microscope.

Table 3. *Nuclear transfer efficiencies for cumulus cells according to model*

	Nuclear Transfer Model			
	Model A	*Model B*	*Model C*	*Model D*
Oocytes				
First manipulation	2,766	1,358	492	104
Survived	1,740	446	304	58
Second manipulation	1,556	122	206	51
Survived	653(23.6)[a]	83(6.1)[b]	112(22.8)[a]	14(13.5)[c]
Cleaved	36(5.5)[a]	30(36.1)[b]	35(31.2)[b]	0(0.0)[c]
Embryos				
Transferred to mice	154	9	30	0
Transferred to rats	269	NA	NA	NA
Developing to morula/blastocyst	29(18.8)[a]	3(33)[a]	0(0.0)[b]	NA
Producing live born	0(0.0)	NA	NA	NA

Values describe overall nuclear transfer efficiency according to model (*A–D*) for donor rat cumulus cells. Values in parentheses are in percent. *Model A,* enucleation of metaphase plate (first manipulation) followed by donor cell injection (second manipulation) followed by chemical activation; *model B,* donor cell injection (first manipulation) followed by chemical activation followed by enucleation of the female pronucleus (second manipulation); *model C,* chemical activation followed by donor cell injection (first manipulation) followed by enucleation of the female pronucleus (second manipulation); and *model D,* chemical activation followed by enucleation of the female pronucleus (first manipulation) followed by donor cell injection (second manipulation). Significant differences in reconstructed embryo survival, cleavage, and development in vivo between models are indicated by different superscript letters (a–c).

(unpublished data). Ethanol/cycloheximide activation also appeared to be of greater utility in the rescue of spontaneously activated oocytes compared with chloral hydrate-induced activation reported by Zernicka-Goetz (32). Although the survival and developmental potential of oocytes rescued with ethanol/cycloheximide following NT procedures was not tested in the present study, this type of chemical activation may provide an efficient means by which to increase the numbers of oocytes available for NT procedures.

To our knowledge, only two reports of rat NT exist (8, 11). In both of those reports, electrofusion was used for introduction of donor nuclear material and activation of the reconstituted oocyte. The present work represents an attempt at NT with chemical activation of both adult somatic cells and genetically modified fetal cells in the Sprague-Dawley rat.

Using Fischer rats (F334/DuCrj), Kono et al. (11) reported high survival rates for NT embryos following electrofusion of embryonic donor DNA into enucleated zygotes (60%, 126/210), high development rates in vitro (73%, 92/126), and live young following embryo transfer (7.5%, 9/120). Our observations with donor DNA obtained from adult somatic cells and injected into the equivalent of zygote cytoplasm (*model D*) indicated lower rates of survival (14.3%) and development to the two-cell stage in vitro (0%) compared with those reported by Kono et al. (11). Overall, cleavage rates for NT embryos reconstructed with cumulus cells were consistently reduced compared with parthenote controls, thus suggesting an effect of procedure independent of the model used. In vivo development of NT embryos reconstructed with cumulus cells was clearly improved with activation of the reconstructed oocyte following donor cell injection (*models A* and *B*) compared with activation prior to donor cell injection (*model C*), despite significant differences in survival and cleavage following micromanipulation. Activation of the recipient cytoplasm 30 min to 1 h after donor cell injection improved in vivo development rates significantly (*models A* and *B*; 32/163, 19.6%) compared with activation of recipient cytoplasm 1–4 h prior to donor

Table 4. *Nuclear transfer efficiencies for transfected fibroblasts according to model*

	Nuclear Transfer Model			
	Model A	*Model B*	*Model C*	*Model D*
Oocytes				
First manipulation	552	493	163	48
Survived	257	94	57	33
Second manipulation	197	53	28	14
Survived	56(10.1)[a]	25(5.1)[b]	18(11.0)[a]	7(14.6)[a]
Cleaved	11(19.6)[a]	5(20.0)[a]	6(33.3)[a]	2(28.6)[a]
Embryos				
Transferred to mice	1(1.8)	1(4.0)	4(22.2)	1(14.3)
Transferred to rats	NA	NA	NA	NA
Developed to morula/blastocyst	0(0.0)	0(0.0)	1(25.0)	0(0)
Producing live born	NA	NA	NA	NA

Values describe overall nuclear transfer efficiency according to model (*A–D*) for donor transfected rat embryonic cells. Values in parentheses are in percent. See legend to Table 3 for complete description of *models A–D*. Significant differences in reconstructed embryo survival, cleavage, and development in vivo between models are indicated by different superscript letters (a and b).

Table 5. *Effect of nocodazole-assisted enucleation and piezoelectric-assisted donor cell injection on nuclear transfer efficiency using cumulus cells and model A*

	Nocodazole[1]/ Mechanical[2]	Nocodazole[1]/ Piezoelectric[2]	Blind[1]/Mechanical[2]	Blind[1]/Piezoelectric[2]
Oocytes				
Enucleated[1]	405	1,212	383	766
Survived	257	746	225	512
Injected[2]	223	675	204	454
Survived	63(15.6)[a]	272(22.4)[b]	89(23.2)[b]	229(29.9)[c]
Cleaved	6(9.5)[a]	19(7.0)[a,c]	1(1.1)[b]	10(4.4)[b,c]
Embryos				
Transferred to mice	25	50	49	30
Transferred to rats	33	109	34	93
Developing to morula/blastocyst	7(28.0)[a]	9(18.0)[a]	8(16.3)[a]	5(16.7)[a]
Producing live born	0(0.0)	0(0.0)	0(0.0)	0(0.0)

Values describe overall nuclear transfer efficiency using nocodazole-assisted enucleation, piezoelectric-assisted donor cell injection, and *model A* for donor rat cumulus cells. Values in parentheses are in percent. The superscript number "1" indicates enucleation, whereas a superscript "2" indicates donor cell injection (i.e., "Nocodazole[1]/Mechanical[2]" indicates nocodazole-assisted enucleation followed by mechanical donor cell injection). Significant differences in reconstructed embryo survival, cleavage, and development in vivo between models are indicated by different superscript letters (a–c).

cell injection (*models C* and *D*; 0/30, 0%). These results indicate that the rat metaphase II oocyte is an environment suitable for reprogramming of donor cells in G_0 and that as little as 1.5–2.0 h of activation prior to donor cell injection may be sufficient to disrupt effective nuclear reprogramming of G_0 cells in this species.

Using inbred Wistar rats, Fitchev et al. (8) reported a survival rate of 10.3% (12/116) for electrofused rat NT embryos reconstructed with transfected fetal fibroblasts, a value consistent with our observations using chemical activation (5–15% survival rates depending on the method employed; Table 4). Despite the similarities in survival rates, we report a recovery rate of 14.3% (1/7) for embryos transferred to recipient mice compared with a recovery rate from recipient rats of 0% (0/5) reported by Fitchev et al. (8). Cleavage rates for NT embryos reconstructed with transfected fibroblasts were consistently higher when activation of the recipient cytoplasm was antecedent with respect to donor cell injection (*models C* and *D*). The only viable embryo recovered of seven transferred was obtained using *model C*. These results differed considerably from the results obtained with cumulus cells (*models A* and *B*) and may reflect the differences in cell-cycle stage of the nuclear donor at the time of activation. However, observations with NT embryos derived from cumulus cells indicated that cleavage alone was not an indicator of developmental potential in vivo (see Table 3, *model C*). Furthermore, the limited numbers of NT embryos derived from transfected fetal cells and transferred to recipients precludes any useful discussion pertaining to the potential limitations or benefits of different NT techniques in relationship to cell-cycle when using this type of donor cell.

Unlike the present study and the study by Fitchev et al. (8), the work by Kono et al. (11) involved transfer of embryonic DNA from donor cells at various stages of development (zygote to 8-cell embryos) into recipient zygote cytoplasm. In that study, the "developmental" match between donor DNA and recipient cytoplasm was clearly important, as enucleated zygotes receiving pronuclear DNA, but not DNA derived from embryonic blastomeres, were capable of full-term development. Fitchev et al. (8) used transfected fibroblasts that were subjected to serum starvation in an attempt to drive the cells into G_0 (9). The serum starvation technique has been used successfully in several species (12, 30), despite widespread disagreement on whether starvation of fibroblasts is an absolute requirement for their full-term development (4, 30). However, Fitchev et al. (8) failed to indicate the duration of serum starvation. Recently, it has been suggested that this type of treatment may fail to drive cells into G_0 because of the heterogeneity of cell states at the time of starvation and the length of starvation (5). Whether variation in cell-cycle stage in response to serum starvation was responsible for a mismatch of donor-recipient cell cycles in the Fitchev study and subsequent failure to recover fetuses from surrogates is not known.

Nocodazole has been shown to disperse the second meiotic spindle of mature rat oocytes and stabilize the metaphase plate as a single, slightly protruded mass (31). The beneficial effects of piezoelectric-assisted injection have been described for NT procedures in the mouse (26). In our experience, a significant proportion of rat oocytes undergo spontaneous abortive activation despite rapid removal from the source animal and maintenance at 37°C, and injection of donor cells can be difficult because of membrane stiffness, despite adequate protein supplement to the medium. In the present study, we have examined the application of nocodazole for mechanical enucleation of the metaphase plate and piezoelectric-assisted donor cell injection on the survival and development of NT embryos constructed with cumulus cells. Metaphase plate enucleation following nocodazole treatment appeared to reduce survival rates but improved cleavage rates of surviving embryos compared with blinded enucleation. In light of the observation that development of cumulus cell-derived NT embryos in mouse oviducts was equivalent with and without nocodazole-assisted enucleation and with and without piezoelectric-assisted

cell injection, it is our submission that blinded enucleation with piezoelectric-assisted cell injection is the most beneficial method for rat NT according to *model A*, because it produced the highest proportion of surviving one-cell embryos (29.9%).

None of the 269 cumulus cell-derived NT embryos developed to term following transfer to the oviducts of pseudopregnant rats. Fitchev et al. (8) reported a decrease in the number of pregnant recipients, and live births per embryo transferred, when one-cell embryos produced by natural mating were transferred to pseudopregnant recipients at 24 h after collection as opposed to 5 h following collection. Those results, like ours using M16 culture medium, were based on early cleavage stage embryos transferred to the oviducts of *day 0* to *day 1* pseudopregnant recipients and suggested some deficiencies in the in vitro culture system and/or timing of pseudopregnancy. Kono et al. (11) have described the birth of live young from NT embryos using M16 as the culture medium and transfer to *day 1* pseudopregnant recipients. However, that report does not indicate whether live births arose from one-cell embryos transferred immediately after fusion or from those embryos cultured for a further 20–24 h in M16. Oh et al. (20) have described the birth of live young produced by in vitro fertilization and culture to the morula/blastocyst stage in a modified rat embryo culture medium and transferred to the uterus of *day 4* pseudopregnant recipients. Clearly, the relationships between culture method, culture duration, and status of NT embryo and recipient are worthy of further investigation, as embryo transfer appears to be a major limitation to the success of NT in the rat.

Here we have described methods for collection, manipulation, and activation of rat oocytes to facilitate the in vivo production of NT embryos reconstructed with adult and genetically modified fetal cells. Mechanical enucleation of the metaphase plate combined with piezoelectric-assisted donor cell injection, along with activation of the reconstructed embryo with ethanol/cycloheximide soon after donor cell injection, appears to be a reliable method for in vivo production of NT morula and/or blastocysts from cumulus cells. Timing of cell injection and reconstructed oocyte activation may be different for NT embryos derived from genetically modified fetal cells. The effects of synchronization of the cell cycle of genetically modified donor cell and recipient ooplasm and the relationships between embryo culture and transfer on developmental outcomes are deserving of further study.

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